fluorescent protein to report target sequence expression and enhanced green fluorescent protein for microRNA expression. Using this assay, we demonstrated a functional target for miR-212 in the 3′ untranslated region of $K_{ir}2.1$. Red/green fluorescence intensity ratio was significantly lower in miR-212-expressing HEK293 cells compared to non-targeting control (miR-212 0.72 ± 0.024 (mean ± sem), n = 550; control 1.21 ± 0.025, n = 731; $p<.001$, log transformed data). The effect of miR-212 was attenuated by mutating the predicted target site (% inhibition 58.0 ± 14.51, n = 3 wild-type; 22.7 ± 1.25, n = 3 mutant). Expression of miR-212 downregulated endogenous $K_{ir}2.1$ protein in HeLa cells, as shown by quantitative western blot of membrane extracts (band intensity *vs* Na^+/K^+-ATPase loading control: miR-212 0.0647 ± 0.0047; non-targeting control 0.0895 ± 0.0045; n=3, $p<.05$). Endogenous inward rectifier K^+ current in HeLa cells was isolated by extracellular application of 100 μM Ba^{2+} during whole-cell patch-clamp recording. Ba^{2+}-sensitive current density was significantly smaller in miR-212-transfected (n=13) *vs* control-transfected cells (n=8); $p<.01$. In conclusion, downregulation of inwardly rectifying K^+ current and $K_{ir}2.1$ expression in heart failure and alcoholic cerebrovascular dysfunction may be functionally linked to upregulation of miR-212.

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Dissecting Gating Rules of GIRK Channels: Role of PIP2 in Ethanol-Dependent Activation

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G protein-gated inwardly rectifying potassium (GIRK) channels are implicated in alcohol abuse and addiction. We discovered a discrete alcohol-binding pocket in the channel mediating ethanol-dependent activation. Here, we investigated the role of G proteins and PIP_2 in ethanol-dependent gating. We engineered GIRK2 with single, modifiable cysteine at L257 in alcohol pocket and found that alcohol-like methanthiosulfonate (MTS) reagents activate GIRK2-L257C, similar to ethanol-dependent activation. We assessed role of G proteins in alcohol activation by either increasing levels of Gβγ (+Gβ1γ2) or decreasing Gβγ through chelation with membrane bound Phosducin (+mPhos). Neither Gβ1γ2 nor mPhos altered the rate of MTS-HE-mediated GIRK2-L257C activation. For comparison, we examined GIRK2-L344C, a key site for Gβγ activation that is inhibited by MTS modification. In contrast to L257C, rate of MTS modification showed a clear dependence on Gβγ levels. These results suggest that alcohol activation of GIRK channel is independent of G proteins. To investigate the role of PIP_2, we used voltage-sensitive phosphatase DR-VSP to transiently deplete PIP_2 in the membrane. Activation of DR-VSP completely reversed MTS-HE activated current of GIRK2-L257C. Furthermore, MTS-HE treatment significantly slowed the rate of GIRK2-L257C current inhibition following DR-VSP activation, suggesting an increase in apparent affinity for PIP_2 and GIRK2-L257C channels modified by MTS-HE. Lastly, we examined the role of PIP_2 on alcohol-dependent activation of wild-type GIRK2. Addition of propanol significantly slowed the rate of wild-type GIRK2 current inhibition following PIP_2 depletion. Taken together, these data demonstrate that alcohol-dependent activation of GIRK involves an increase in apparent affinity for PIP_2, with little influence of Gβγ subunits. The fundamental dichotomy between alcohol and Gβγ arises from distinct gating mechanisms converging on PIP_2-dependent opening, revealing novel pathways for antagonizing alcohol's effects on ion channels.

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Unique PIP2 Sensitivity at a Putative PKC Site in GIRK2 (Kir3.2)

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G-protein activated inward rectifier potassium channels (GIRKs) exhibit sensitivity to a diverse range of modulators, including G-proteins, sodium, PIP_2, and phosphorylation by PKA and PKC. The residue Ser-196 in the GIRK2 subunit is implicated in PKC sensitivity in the homologous GIRK1 and GIRK4 subunits. It is located distal to the helix bundle crossing, and is situated to interact with both Phe-192 of the bundle crossing and Thr-317 of the G-loop gate. In the background of the highly active homomeric GIRK2 mutant E152D, we mutated Ser-196 to Ala and tested its PIP_2 dependence, using the voltage-sensitive PIP phosphatase Ci-VSP. The S196A mutant was inhibited normally by activation of Ci-VSP, but upon recovery it displayed a unique behavior. Instead of a monophasic recovery, the S196A mutant exhibited a characteristic inhibition following recovery, which was not observed in the homomeric mutant alone. In addition, the S196A mutant current recovery depended on the initial level of PIP_2 depletion. Mutant channels S196E and S196Q did not reproduce the unique pattern of S196A. Using the G_q-coupled hM1 assay, we tested the muscarinic sensitivity of S196A vs. homomeric mutant. While the homomeric mutant was inhibited normally, the S196A channel did not show appreciable inhibition. Taken together, these results indicate that the S196A mutant exhibits unique PIP_2 sensitivity. Given the critical location of S196 to the channel gates we are pursuing the structural mechanism that could explain the unique behavior of the Ala mutant.

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HDAC Inhibitors Affect Sulfonylurea Receptor Subunit MRNA Expression in Atrial-Derived Hl-1 Cells but not Pancreatic Beta Cell-Derived MIN6 Cells

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K_{ATP} channels are expressed in many types of excitable cells where they typically act as key sensors of cell metabolism. All K_{ATP} channels share the same architecture—a K^+ channel pore (Kir6.1 or Kir6.2) combines with a sulfonylurea receptor (SUR1, SUR2A or SUR2B) to form a functional channel. Importantly, K_{ATP} channels composition is tissue specific. SUR1 and Kir6.2 make up the channel in atrial cardiomyocytes and pancreatic beta cell, while SUR2A combines with Kir6.2 to form ventricular myocyte K_{ATP}. Tissue specific heterogeneity appears to be driven principally by differential subunit transcription, but the mechanisms that determine when and where specific K_{ATP} channels are expressed are poorly understood. In this study, we have employed both cardiac- (HL-1) and pancreatic beta cell- (MIN6) derived cell lines to explore the mechanisms that control SURx gene expression. In both HL-1 and MIN6 cells we find that SUR1 expression is significantly greater than SUR2. When cells are treated for 72 hours with trichostatin A (a general inhibitor of histone deacetylases or HDACs), there is a significant increase in SUR2 subunit expression in HL-1 cells, but no apparent change in SUR2 expression in MIN6 cells. This result indicates that in the absence of HDAC activity, the transcriptional machinery to drive SUR2 gene expression is available in HL-1, but not in MIN6 cells. From this data, we conclude that both the SURx subunit transcriptional profile and the mechanisms that determine that profile are tissue specific.

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Lessons from KATP Channels with Diabetogenic Mutations in Sulfonylurea Receptor 1 (SUR1)

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Numerous mutations have been identified in SUR1 (ABCC8) subunit of the neuroendocrine type KATP channel in subjects with neonatal diabetes, neonatal diabetes plus epilepsy and/or other neurological features, maturity-onset diabetes of young, and later-onset diabetes. Patch-clamping, single-channel kinetics analysis, affinity photolabeling and molecular modeling were used to clarify how diabetogenic mutations in different parts of SUR1 affect open probability and sulfonylurea inhibition of SUR1/Kir6.2 KATP channel. Essentially all tested diabetogenic mutations in the canonical TMD1-NBD1-TMD2-NBD2 ABC exporter core of SUR1 hyperactivated KATP by stabilizing the stimulatory Mg-nucleotide bound (outward facing) state of SUR1 without affecting the intrinsic gating of KATP channel or its sensitivity to inhibitory nucleotides. Hyperstimulated mutant channels showed attenuated sulfonylurea inhibition in the presence, but not the absence, of stimulatory MgATP/ADP, indicating that KATP with SUR1 in the inward facing state has the lowest Kd for sulfonylureas. Diabetogenic mutations in the non-canonical TMD0-L0 part of SUR1 hyperactivated KATP by destabilizing its long-lived closed state with the highest affinity to inhibitory ATP or by strengthening the functional coupling between the MgATP/ADP-bound SUR1 core and the active (burst) state of the Kir pore. Mutations destabilizing the long-lived closed state compromised sulfonylurea inhibition of KATP in the absence of nucleotides but not the drug-induced release of stimulatory nucleotides. The findings support the mechanistic model (FEBS Letters, 585:3555-9) in which the TMD0-L0 module couples the SUR1 core with the KATP pore, define the most common ABCC8-associated mechanisms of KATP hyperactivity, and largely explain why the majority of diabetic subjects with mutant SUR1 require body-weight normalized doses of sulfonylureas exceeding those recommended by the FDA for treatment of common type 2 diabetes.

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A Single Point Mutation in the Distal C-Terminal of the Pore Forming Kir6.1 Subunit Modifies ATP-Sensitive Potassium (K_{ATP}) Channel Regulation

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